Angiopoietin-2 is associated with decreased endothelial nitric oxide and poor clinical outcome in severe falciparum malaria

Tsin W. Yeo[a,b], Daniel A. Lampah[c,d], Retno Gitawati[e], Emiliana Tjitra[e], Enny Kenangalem[c,d], Kim Piera[a,b], Ric N. Price[a,b,f,g], Stephen B. Duffull[h], David S. Celermajer[i,j], and Nicholas M. Anstey[a,b,f,1]

[a]International Health Division, Menzies School of Health Research, [b]Charles Darwin University, Darwin NT 0810, Australia; [c]Menzies School of Health Research, National Institute of Health Research and Development Research Program, [d]District Ministry of Health, Timika, Papua, Indonesia; [e]National Institute of Health Research and Development, Jakarta 10560, Indonesia; [f]Division of Medicine, Royal Darwin Hospital, Darwin NT 0810, Australia; [g]Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford OX3 7LJ, United Kingdom; [h]School of Pharmacy, University of Otago, Dunedin 9054, New Zealand; [i]Department of Medicine, University of Sydney, Sydney NSW 2006, Australia; and [j]Department of Cardiology, Royal Prince Alfred Hospital, Sydney NSW 2006, Australia

Edited by Thomas E. Wellems, National Institutes of Health, Bethesda, MD, and approved September 23, 2008 (received for review June 15, 2008)

Adherence of parasitized erythrocytes to activated endothelium causes microvascular obstruction, tissue ischemia, and clinical complications in severe malaria (SM); however, the mechanisms leading to endothelial activation remain unclear. The angiogenic factors, angiopoietin-2 (Ang-2) and vascular endothelial growth factor (VEGF) are modulators of endothelial activation, with Ang-2 release from Weibel–Palade bodies (WPBs) being regulated by endothelial nitric oxide (NO). We explored the relationships between endothelial NO bioavailability, Ang-2, VEGF, tissue perfusion, and clinical outcomes in SM. We measured plasma Ang-2 and VEGF, together with biomarkers of severity from 146 adults with and without SM, in parallel with longitudinal measures of endothelial function by using reactive hyperemia peripheral arterial tonometry (a measure of endothelial NO bioavailability). Regression was used to relate concentrations of Ang-2/VEGF with malaria disease severity, biomarkers of perfusion, endothelial activation, and parasite biomass. The longitudinal relationship between Ang-2 and endothelial function was assessed by using a mixed-effects model. Ang-2 concentrations were elevated in SM and associated with increased venous lactate, plasma intercellular cell adhesion molecule-1 concentrations, parasite biomass, and mortality. In contrast, VEGF concentrations were inversely associated with these biomarkers. Ang-2 concentrations were significantly better predictors of death than venous lactate ($P = 0.03$). Recovery of endothelial function was associated with falling concentrations of Ang-2. Ang-2 release from endothelial cells with reduced NO bioavailability is likely to contribute to endothelial activation, sequestered parasite biomass, impaired perfusion, and poor outcome in severe falciparum malaria. Agents that improve endothelial NO, reduce WPB exocytosis, and/or antagonize Ang-2 may have therapeutic roles in SM.

Plasmodium falciparum | VEGF | Weibel-Palade bodies | endothelial function

The central process in the pathogenesis of severe and fatal falciparum malaria is microvascular obstruction resulting from cytoadherence of parasitized red cells to activated endothelial cells, associated with impaired bioavailability of endothelial nitric oxide (NO). Clinical studies in both adults and children demonstrate microcirculatory obstruction in vivo (1, 2) and impaired vasomotor function (3). These result in decreased oxygen delivery, tissue hypoxia, and metabolic acidosis in severe malaria (SM) (4). Similarly, postmortem studies demonstrate sequestration of parasitized erythrocytes in the capillaries and postcapillary venules of multiple organs (5, 6), which bind and colocalize with the inducible endothelial adhesion receptor intercellular cell adhesion molecule-1 (ICAM-1) (7). Plasma concentrations of adhesion receptors, including ICAM-1 and E-selectin (7, 8) and other markers of endothelial activation, such as von Willebrand factor (VWF) propeptide and microparticles are also markedly increased in SM (9, 10).

Although these findings suggest that endothelial activation is a central process in the pathogenesis of malaria, mechanisms underlying endothelial activation in malaria are incompletely understood. Parasitized erythrocytes and elevated levels of proinflammatory cytokines in malaria are thought to increase the synthesis and expression of endothelial adhesion receptors (11, 12). Recent data suggest that exocytosis of Weibel–Palade bodies (WPBs) from endothelial cells may also be important in endothelial activation in malaria, in the early phase of adult experimental infection before levels of proinflammatory cytokines are high enough to cause clinical disease (13) and in African children with severe disease (9).

The angiogenic factors, angiopoietin-2 (Ang-2) and vascular endothelial growth factor (VEGF), have recently been shown to regulate the vascular inflammatory response (14, 15), with plasma levels increased in severe sepsis (15, 16). Ang-2 is stored and released from WPBs (17), functioning as an autocrine regulator by sensitizing the endothelium to the effects of tumor necrosis factor (TNF), resulting in increased adhesion receptor expression (18). In vitro, VEGF increases the expression of ICAM-1 and Ang-2 in endothelial cells (19, 20). Although plasma VEGF concentrations have been associated with an increased risk of neurologic sequelae in African children with cerebral malaria (21), the role of Ang-2 in malaria pathogenesis is unknown.

In vitro, endothelial NO is the major inhibitor of the release of WPB contents (22). We have recently shown that endothelial function, a measure of vascular NO bioavailability (23), is significantly impaired in SM in adults (3). To our knowledge, the relationship between endothelial dysfunction and WPB exocytosis in malaria, or indeed any infectious disease, is unknown. We hypothesized that concentrations of Ang-2 and VEGF would be associated with endothelial activation and malaria disease severity, and that the decreased vascular NO bioavailability in malaria may contribute to an increase in Ang-2. We therefore measured plasma Ang-2 and VEGF concentrations in adults with and without SM and examined their relationship with endothelial function and activation, parasite biomass, and clinical outcome.

Results

Patients. A total of 146 adults were prospectively enrolled in Papua, Indonesia between February 2005 and February 2006 (3). Of these

Author contributions: T.W.Y., R.G., E.T., S.B.D., D.S.C., and N.M.A. designed research; T.W.Y., D.A.L., E.T., E.K., K.P., and N.M.A. performed research; T.W.Y., R.N.P., and N.M.A. analyzed data; and T.W.Y., R.G., R.N.P., D.S.C., and N.M.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: anstey@menzies.edu.au.

Table 1. Baseline characteristics of patients according to clinical status

Characteristic	HC	MSM	SM
Number	18	77	51
Age, mean (range), y	25 (18–44)	28 (18–56)	29 (18–56)
Males, no. (%)	13 (74)	32 (67)	37 (72)
Weight, mean (range), kg	59 (45–73)	58 (43–77)	57 (45–85)
Ethnicity, no. (%) Papuan highlander*	15 (87)	59 (77)	27 (53)
Current smoker, no. (%)	8 (45)	31 (40)	22 (43)
Days of fever before presentation; median (IQR)†	0	2 (1–5)	4 (1–7)
Systolic blood pressure; mean (range), mmHg†	128 (96–136)	114 (88–152)	106 (60–154)
Pulse rate; mean (range), beats/minute†	66 (44–104)	86 (56–118)	97 (61–138)
Respiratory rate; mean (range), breaths/min†	20 (18–26)	25 (14–42)	30 (16–60)
Temperature; mean (range), ^{0} C†	35.6 (35–36.7)	36.5 (34.1–39.8)	37.1 (34.8–40.3)

*, $P < 0.01$ calculated by χ^2 test. †, $P < 0.01$ calculated by ANOVA or two-sided t test.

participants, 51 had SM, 77 had moderately SM (MSM), and 18 were healthy controls (HC). SM was defined as the presence of *Plasmodium falciparum* parasitemia and ≥1 modified World Health Organization (WHO) criterion of severity (excluding severe anemia) (24); and MSM was defined as fever within the preceding 48 h, with >1,000 asexual *P. falciparum* parasites/μL, with no WHO warning signs or criteria for SM and a requirement for inpatient parenteral therapy because of an inability to tolerate oral treatment (3, 25). In patients with SM, 28 (55%) had coma, 17 (33%) had acute renal failure, 23 (45%) had hyperbilirubinemia with either renal impairment or parasitemia >100,000/μL, and 30 (59%) had >1 criterion for severe disease. In total, 35 (69%) patients with SM were treated with artesunate and 16 (31%) with quinine, whereas all but 1 of the 77 MSM patients were treated with quinine, the other receiving artesunate. There were 8 (16%) deaths among SM patients and none in the MSM group. Baseline characteristics of the 146 patients are shown in Table 1.

Ang-2, VEGF, and Clinical Disease. Concentrations of Ang-2 were 2.8-fold higher in patients with SM [15,000 pg/mL; interquartile range (IQR): 6,300–22,000] than those with MSM (5,300 pg/mL; IQR: 3,900–8,200) and 6.4-fold higher than HC (2,300 pg/mL; IQR: 1,900–3,600; $P < 0.001$) (Fig. 1*A* and Table 2). Ang-2 was also significantly increased in patients with MSM compared with HC ($P < 0.001$) (Fig. 1*A*). Conversely VEGF concentration was significantly lower in patients with SM (51 pg/mL; IQR: 15–115) compared with patients with MSM (86 pg/mL; IQR: 54–127) and HC (89 pg/mL; IQR: 60–133; $P = 0.006$), with no significant difference in VEGF between the MSM and HC groups (Fig. 1*B* and Table 2). Ang-2 was negatively correlated with VEGF ($r = -0.32$; $P < 0.001$) in all groups.

Patients with cerebral malaria ($n = 14$) and hyperbilirubinemia ($n = 3$) as the sole presentations had Ang-2 concentrations of 5,000 pg/mL (IQR: 3,900–7,000) and 9,800 pg/mL (range: 9,700–17,000), respectively. These concentrations increased significantly with progressive organ dysfunction, with Ang-2 concentration in patients with 2 severity criteria ($n = 26$) rising to 25,000 pg/mL (IQR: 16,000–37,000) and 43,000 pg/mL (IQR: 37,000–49,000) in those with 4 severity criteria ($n = 4$; $P = 0.02$). There was no correlation between VEGF concentrations and number of severity criteria.

In patients with SM who survived there was a mean decrease in Ang-2 during recovery of 2,700 pg/mL per 24 h (95% C.I., 1,600–3,800; $P < 0.001$) (Fig. 2*A*). In contrast, 3 of 8 fatal cases who had repeat measurements of Ang-2 at 24–48 h all showed an increase (mean 9,700 pg/mL (95% C.I., 1,400–33,000) compared with the admission value, which was significantly different from survivors ($P = 0.007$). VEGF was not correlated with an increased risk of death.

Ang-2, VEGF, and Biomarkers of Severity. Patients with SM had higher concentrations of lactate (2.89 mmoL/L; 95% C.I.: 2.34–3.44) compared to patients with MSM (1.36 mmoL/L; 95% C.I., 1.17–1.55; $P < 0.0001$; Fig. 1*C*), elevated creatinine (282 μmoL/L; 95% C.I., 205–360 vs. 84 μmoL/L; 95% C.I., 78–89; $P < 0.001$), as well as increased soluble ICAM-1 (937 pg/mL; 95% C.I., 795–1,080 vs. 518 pg/mL; 95% C.I., 470–566; $P < 0.0001$) and E-selectin (152 pg/mL; 95% C.I., 113–192 vs. 106 pg/mL; 95% C.I., 95–118; $P = 0.009$; Table 2). As the peripheral parasite count does not accurately reflect the sequestered parasite load, we measured *P. falciparum* histidine-rich protein-2 (HRP2) to quantitate total parasite biomass as described (26). Plasma HRP2 was significantly higher in patients with SM compared with those with MSM ($P < 0.0001$; Table 2).

In all patients with malaria, increasing Ang-2 was associated with increased lactate ($r = 0.57$; $P < 0.001$), creatinine ($r = 0.74$; $P < 0.001$), ICAM-1 ($r = 0.69$; $P < 0.001$), E-selectin ($r = 0.5$; $P < 0.001$), and HRP2 ($r = 0.64$; $P < 0.001$), and these associations remained significant after patients were stratified by disease severity (Table 3). There was no association between peripheral parasitemia and Ang-2. The association between lactate and Ang-2 remained significant after adjusting for age, sex, ethnic group, weight, and factors shown to affect lactate concentration, including base deficit, bicarbonate, creatinine, ICAM-1, reactive hyperemia peripheral arterial tonometry (RH-PAT) index, and parasite biomass (3, 4, 26). Similarly, ICAM-1, E-selectin, and HRP2 remained significantly associated with Ang-2 after adjustment for these factors.

Increasing VEGF concentrations were associated with a decrease in blood lactate ($r = -0.39$; $P < 0.001$), HRP2 concentrations ($r = -0.34$; $P < 0.001$), E-selectin ($r = -0.28$; $P = 0.03$), and Ang-2 ($r = -0.32$; $P = 0.001$), and they remained significant after stratifying by disease severity and adjustment for confounding factors (Table 3).

Ang-2 and TNF. TNF was measured only in patients with SM, and the median plasma concentration was 2.4 pg/mL (IQR: 1.5–4.2); 9/51 (18%) had plasma TNF concentrations ≥5 pg/mL, the lowest concentration in vitro at which TNF is able to activate endothelium when combined with 2,000 pg/mL Ang-2 (18). Only 2/51 (4%) had TNF concentrations ≥20 pg/mL, the level at which TNF alone is able to activate endothelium in vitro (albeit less than the activation achieved when combined with Ang-2) (18), and only 1/51 (2%) had a concentration ≥40 pg/mL, the level in vitro at which TNF activation of endothelium is independent of Ang-2 (18). There was a significant association between TNF and Ang-2 ($r = 0.64$; $P < 0.001$), plasma ICAM-1 ($r = 0.44$, $P = 0.009$), and plasma HRP2 ($r = 0.56$; $P < 0.001$) concentrations, but not with lactate ($P = 0.29$). The associations between Ang-2 and lactate, ICAM-1, and parasite biomass remained significant after adjusting for the effects of TNF.

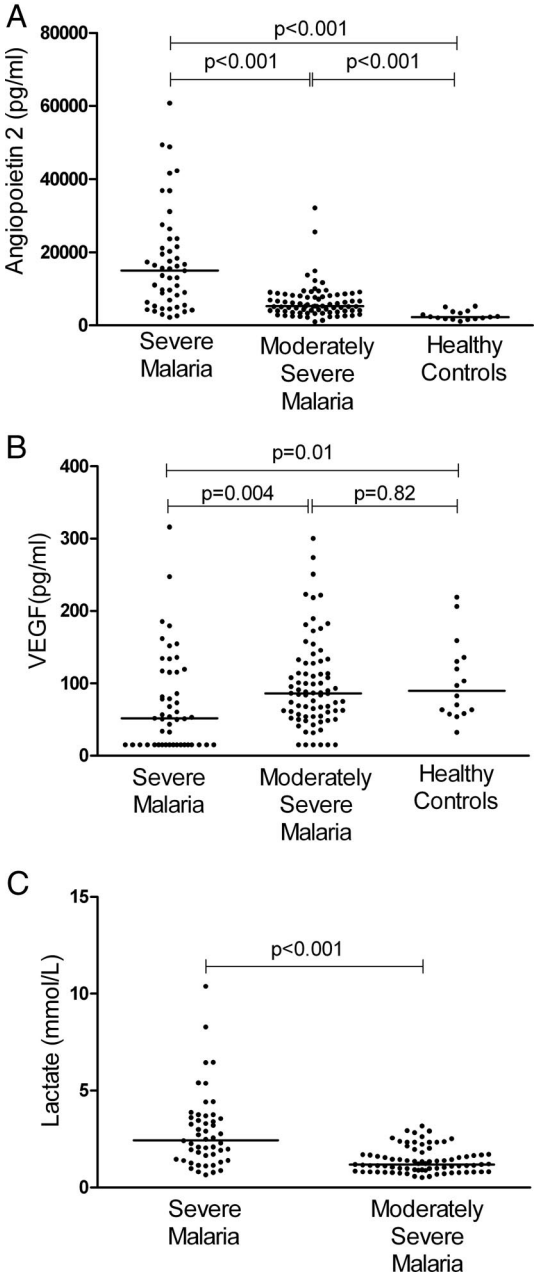

Fig. 1. Ang-2, VEGF, and lactate concentrations in patients with MSM and SM and HC. (*A*) Ang-2 plasma concentrations among disease categories (Kruskal–Wallis: $P < 0.001$). (*B*) VEGF plasma concentrations among disease categories (Kruskal–Wallis: $P = 0.006$). (*C*) Lactate concentrations ($P < 0.001$). Horizontal lines indicate median for each group. Differences among groups were compared by using the Kruskal–Wallis test. The Mann–Whitney test was used for post hoc pairwise comparisons.

In addition, there was no evidence of an effect of the interaction term between Ang-2 and TNF on these markers of disease severity.

Endothelial Function and Ang-2. The mean RH-PAT index was significantly lower in the SM group (1.41; 95% C.I., 1.33–1.47; $n = 49$) than the MSM group (1.82; 95% C.I., 1.71–1.93; $n = 77$) and HC (1.94; 95% C.I., 1.68–2.09; $n = 18$) ($P < 0.0001$). At enrollment, among all patients with malaria there was a negative correlation between RH-PAT index and Ang-2 concentrations ($r = -0.36$; $P < 0.001$); however, this finding was not significant after stratifying by disease severity. In patients with SM, there was a significant longitudinal association between the increase in RH-PAT index (as a measure of endothelial NO bioavailability) (27) and decreasing Ang-2 concentrations ($r = -0.38$; $P < 0.001$) (Fig. 2). There was a positive association between VEGF and RH-PAT index ($r = 0.30$; $P = 0.01$), which was not significant when stratified by disease severity.

Effect of Ang-2 on Mortality in SM. In patients with SM, the Ang-2 concentrations were higher in nonsurvivors (24,000 pg/mL; IQR: 21,000–42,000) compared with survivors (11,000 pg/mL; IQR: 5,000–18,000; $P = 0.004$). Plasma Ang-2 was associated with an increased risk of death (odds ratio 4.9; 95% C.I., 1.3–18.4); other univariate risk factors are listed in Table 4. In a multivariable analysis of variables hypothesized to contribute to mortality and endothelial pathology, Ang-2, HRP2, and the term representing the interaction between Ang-2 and HRP2 all remained significantly associated with increased risk of death ($r = 0.67$).

Receiver Operating Curve (ROC) Characteristics. We compared the areas under the ROC (AUROC) to assess the prognostic value of the independent variables in assessing a fatal outcome in patients with SM. Ang-2 (AUROC 0.84; 95% C.I., 0.71–0.96) (Fig. 3) concentrations were significantly better predictors of death than venous blood lactate (AUROC 0.63; 95% C.I., 0.41–0.83; $P = 0.03$) and comparable to base deficit (AUROC 0.73; 95% C.I., 0.53–0.92), TNF (AUROC 0.71; 95% C.I., 0.43–0.98), and HRP2 (AUROC 0.86; 95% C.I., 0.73–0.94).

Discussion

This study highlights the important finding of increased Ang-2 concentrations in patients with SM and its association with tissue ischemia (reflected as elevated lactate levels), endothelial activation, parasite biomass, organ dysfunction, and death. Blood lactate and base deficit have been described to be reliable prognostic indicators of increased mortality in adults with malaria (4, 26). In predicting a fatal outcome, Ang-2 was comparable to acidosis and parasite biomass and significantly better than lactate, even after controlling for parasite biomass. Among survivors of SM, recovery of endothelial NO bioavailability was associated with falling concentrations of Ang-2.

Proteins stored within endothelial cell WPBs, including Ang-2, VWF and its propeptide, P-selectin, and endothelin-1 are rapidly released in response to stimuli including thrombin, histamine, VEGF, superoxide anions, epinephrine, and vasopressin, and acutely regulate vascular functions such as inflammation, thrombosis, and vasoconstriction (28). These proteins rapidly effect changes in the endothelium, in contrast to the modulation of gene expression effected by proinflammatory cytokines, which requires several hours (29). Ang-2 is almost entirely produced by the endothelium in WBPs, where it is stored with VWF, but not P-selectin (17). It acts as a rapid autocrine regulator of endothelial inflammation (17) and factors controlling its transcription include cytokines such as VEGF and hypoxia (20). The time course of the increase in VWF in experimental malaria (13) suggests that Ang-2 release has a potential role in endothelial activation early in the course of disease. Ang-2 levels were increased in MSM relative to controls, demonstrating WPB exocytosis and endothelial activation in nonsevere clinical malaria. Factors triggering Ang-2 release from WPBs early in the course of malaria are not known. In vitro, exocytosis of WBPs can be triggered by Toll-like receptor (TLR) 2 and TLR4-mediated signaling pathways (30, 31), but not by direct stimulation with TNF (17, 32). *P. falciparum* glycosylphosphatidylinositol released during erythrocyte rupture signals via both TLR2 and TLR4 (33), and along with intact infected red blood cells, can directly activate endothelial cells in vitro; however, their effects on WPB release have not been described (34, 35). *P. falciparum* secretes a functional histamine releasing factor homologue and it is possible that this may also contribute to exocytosis of WPBs (36).

Table 2. Baseline laboratory and physiological measurement according to clinical status

Measurement	HC	MSM	SM
Number	18	77	51
White blood cell count, mean (95% C.I.), $\times 10^3 \mu L^{-1}$*	ND	5.9 (2.6–10.8)	9.4 (3.2–17.3)
Hemoglobin, mean (range), g/L*	ND	128 (7–17)	108 (6–16.3)
Plasma Ang-2 median (IQR), pg/mL*	2,300 (1,900–3,600)	5,300 (3,900–8,200)	15,000 (6,300–22,000)
Plasma VEGF median (IQR), pg/ml*	89 (60–133)	86 (54–127)	51 (15–115)
Plasma creatinine, mean (95% C.I.), μmol/L*	ND	84 (78–89)	282 (205–360)
Lactate concentration, mean (95% C.I.), mmol/L*	ND	1.4 (1.2–1.6)	2.89 (2.3–3.4)
Parasite density, geometric mean (range), μL^{-1}*	ND	14,345 (850–127,350)	35,067 (125–725,340)
HRP2 concentration, mean (range), $\log_e$ ng/mL*	ND	5.75 (1.02–8.79)	7.53 (1–10.98)
Soluble ICAM-1, mean (95% C.I.), pg/mL*	ND	569 (516–623)	937 (795–1,080)
Soluble E-selectin, mean (95% C.I.), pg/mL*	ND	106 (95–118)	152 (113–192)
RH-PAT index, mean (95% C.I.)*	1.94 (1.68–2.09)	1.82 (1.71–1.93)	1.41 (1.33–1.47)

ND, not measured. *, $P < 0.01$ calculated by ANOVA/Kruskal–Wallis or Mann–Whitney/two sided *t* test..

Tie-2 protein kinase receptors are expressed primarily on endothelial cells, and this signaling pathway reduces endothelial activation (14). Ang-2 binds to Tie-2 receptors without activating the signaling pathway, resulting in increased endothelium adhesion receptor expression (14). In vivo, TNF inoculation into Ang-2 knockout mice causes a reduction in endothelial adhesion receptor expression and leukocyte adhesion compared with wild types (18). In vitro studies of human endothelial cells demonstrate that alone neither Ang-2 and TNF at low concentrations (2,000 pg/mL and ≤5 pg/mL, respectively) increase ICAM-1 expression, but together result in a marked elevation in ICAM-1 receptors even at low concentrations (18). In vitro, TNF concentrations >40 pg/mL are required to induce endothelial activation independently of Ang-2 (18), a concentration found in plasma in only 2% of SM patients in our study. In a previous study of 287 adults with SM, 77% had TNF concentrations <15 pg/mL, with the median value being 0 pg/mL and the IQR being 0–4 pg/mL (37). It is likely that local microvascular concentrations of TNF in the vicinity of erythrocyte rupture may be higher than those found in peripheral blood in SM. In addition, the concentrations of Ang-2 we have found in clinical malaria are higher than those used for in vitro studies (18) and may sensitize the endothelium to lower levels of TNF. Taken together, the in vitro and in vivo findings suggest that Ang-2 acts by sensitizing endothelial cells to respond to levels of TNF that may otherwise cause only minimal or no endothelial activation.

In SM we found Ang-2 was associated with endothelial activation, lactic acidosis, increased creatinine, parasite biomass, and mortality, even after adjusting for the effects of TNF. These findings are consistent with Ang-2 sensitizing the endothelium across a range of TNF concentrations (18). Because increased endothelial activation exacerbates sequestration of parasitized red cells and microvascular obstruction, subsequent deterioration in tissue hypoxia would increase transcription and fuel further release of Ang-2 with increasing disease severity. Ang-2 is thus likely to have a regulatory role on endothelial activation from preclinical malaria to severe disease.

In vitro, the only substances demonstrated to reduce exocytosis of WPBs and release of Ang-2 are NO and high doses of hydrogen peroxide (22, 29). We hypothesized that the decrease in NO bioavailability previously described in SM in both adults (3, 27) and children (38) may contribute to increased Ang-2 release. Because the RH-PAT index is at least 50% dependent on endothelial NO production (39), the longitudinal finding of an inverse association between RH-PAT index and Ang-2 suggests that, similar to in vitro

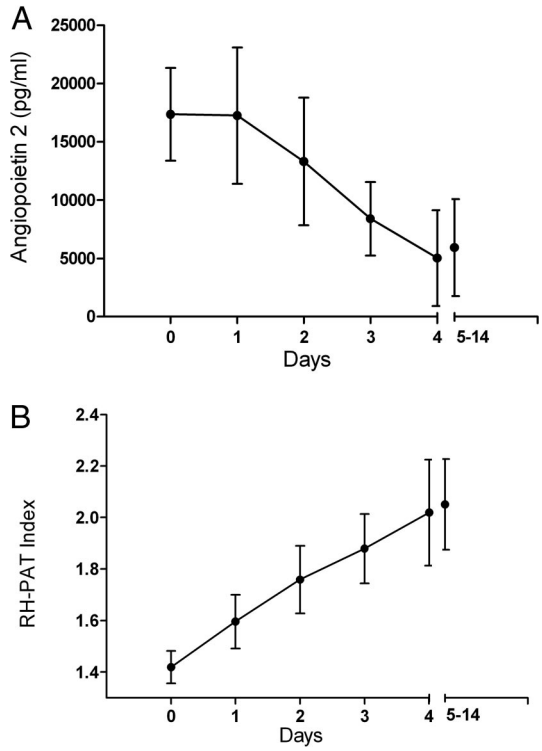

Fig. 2. Longitudinal course of plasma Ang-2 concentrations (*A*) and RH-PAT index (*B*) over time. Mean values (circles) and 95% C.I. (bars) are displayed at each time point. *x* axis values show time from the start of antimalarial therapy (day 0, 0–12 h; day 1, 13–36 h; day 2, 37–60 h; day 3, 61–84 h; day 4, 85–109 h; day 5–14, >110 h). In a mixed-effects model, the increase in RH-PAT index (27) was associated with the fall in Ang-2 ($r = -0.38$; $P < 0.001$).

Table 3. Correlation coefficients (R_s) for biomarkers of severity in 126 patients with MSM and SM

Angiogenic factor	Biomarker	Correlation, *r*	*P*	df
Ang-2	VEGF	0.32	0.001	125
	Creatinine	0.74	< 0.001	124
	Lactate	0.57	< 0.001	124
	HRP2	0.64	< 0.001	118
	ICAM-1	0.69	< 0.001	118
	E-selectin	0.5	< 0.001	115
VEGF	Creatinine	0.18	0.21	124
	Lactate	0.39	< 0.001	124
	HRP2	0.34	< 0.001	118
	ICAM-1	0.17	0.067	118
	E-selectin	0.28	0.038	115

Table 4. Factors associated with an increased risk of death in SM

Factor	Odds ratio (95% C.I.)	*P*	df
Ang-2 (log increase)	4.9 (1.3–18.4)	0.018	49
Creatinine (log increase)	8.6 (2–36)	0.003	50
HRP2 (log increase)	3.7 (1.3–10.3)	0.015	47
Base deficit	1.2 (1.1–1.7)	0.03	50
Lactate	3.7 (0.8–5.3)	0.18	50

data, reduced endothelial NO bioavailability may contribute to or exacerbate endothelial Ang-2 release in severe disease.

It is possible that other substances, including VWF, copackaged in WPBs with Ang-2, may also contribute to endothelial activation after exocytosis of WPBs. However, whereas VWF propeptide has been associated with increased lactate in African children with falciparum malaria, no relationship was found between VWF or P-selectin with either lactate or mortality (9). P-selectin is also contained within WPBs and contributes to parasite cytoadherence in vitro and increased mortality in murine models (40, 41), but its role in clinical disease is unclear with two studies showing no association between plasma P-selectin concentrations and disease severity (9, 42). Furthermore, because Ang-2 and P-selectin are localized to different parts of the WPBs and their release appears to be independent of each other (17), the associations we have found with Ang-2 are unlikely to be related to P-selectin.

VEGF is another angiogenic factor able to increase endothelial activation in addition to increasing vascular permeability (19). In African children with cerebral malaria, plasma VEGF concentrations have been associated with an increased risk of neurological sequelae (21), but there have been no reports comparing VEGF concentrations in cerebral malaria with levels in children with less severe disease (21). In contrast to our initial hypothesis, we found no association between elevated VEGF concentrations and disease severity or a poor outcome in adult malaria, similar to recent findings by Jain *et al.* (43). Indeed, VEGF concentrations were lowest in SM and inversely associated with lactate. These unexpected results may be explained by the recent finding that mature *P. falciparum* parasites, sequestered within the microvasculature, take up and accumulate host VEGF within the parasitophorous vacuole (44), resulting in the likely removal and sequestration of circulating VEGF. This notion is supported by our finding of an inverse association between plasma VEGF concentrations and parasite biomass. Accumulation of VEGF within parasites acts as a parasite growth factor (44) and may exacerbate parasite sequestration. VEGF increases endothelial production of NO (45) and Ang-2 (20) in vitro. It is not known whether accumulation of VEGF within sequestered parasites exerts local effects on adjacent host endothelial cells through these mechanisms. Alternatively, impairment of circulating VEGF suggests a potential contribution to the impaired endothelial NO production and endothelial dysfunction described in SM (3, 27).

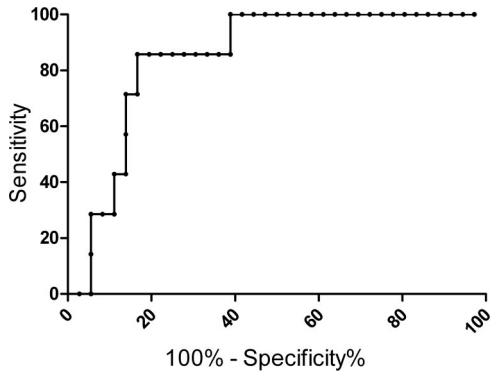

Fig. 3. Nonparametric ROC assessing Ang-2 as a prognostic marker for mortality in SM. AUROC, 0.84; 95% C.I., 0.71–0.96.

Limitations of our study include the inability to establish causality from the associations between Ang-2 and disease severity and outcome. It is possible that increasing parasite biomass and proinflammatory cytokines cause both lactic acidosis and mortality, with worsening hypoxia increasing Ang-2 production as an epiphenomenon. However, in a multivariable model to examine the effects of Ang-2, cytokines, and parasite biomass to predict mortality, TNF was not predictive and Ang-2 remained a significant independent predictor after including both HRP2 and the Ang-2–HRP2 interaction term. We hypothesize a synergistic and potentially bidirectional effect of Ang-2-related endothelial activation promoting sequestration of parasitized red cells and microvascular obstruction, with a resulting hypoxia-induced increase in Ang-2 transcription. Neither children nor patients with severe malarial anemia were included in this study, and we cannot extend our results to these groups. Because of some differences between Asia–Pacific adults and African children in SM phenotypes (46), rapidity of disease progression and TNF concentrations in SM (37, 38, 47), additional studies of the role of Ang-2 in the African pediatric population are needed. Nevertheless, as in adults, NO deficiency (38), endothelial activation (8), and WPB release (9) are also associated with malaria disease severity in African children, making it plausible that Ang-2 will contribute to malaria pathogenesis in children.

In summary, concentrations of plasma Ang-2, an autocrine regulator of endothelial activation, are increased in SM and associated with increased mortality, and with measures of endothelial activation, sequestered parasite biomass and impaired perfusion. We hypothesize that the decreased vascular NO bioavailability found in SM increases exocytosis of WPBs with a resultant increase in Ang-2 release. Agents that improve endothelial NO, reduce WPB exocytosis (48), or inhibit Ang-2 (49) may have preventative or therapeutic roles in SM.

Methods

Study Design and Site. This prospective longitudinal study was conducted at Mitra Masyarakat Hospital, Timika, Papua, Indonesia, an area with unstable transmission of multidrug-resistant falciparum and vivax malaria (50, 51). Ethical approval was obtained from the Health Research Ethics Committees of the National Institute of Health Research and Development in Indonesia and Menzies School of Health Research in Australia. Written informed consent was obtained from patients or relatives.

Patients and Sampling. Patients were 18 years or older hospitalized with MSM or SM without concurrent *P. vivax* infection and with a hemoglobin level >60 g/L, who had been prospectively enrolled in a study of endothelial function as described (3, 27). Individuals with MSM were treated with i.v. quinine whereas SM patients received either i.v. quinine or artesunate, with both groups also receiving either doxycycline or clindamycin. On enrollment, a standardized history and clinical examination were obtained either from the patient or attendant relatives together with collection of venous blood and measurement of endothelial function. Patients were re-examined daily until death, discharge from hospital, or their endothelial function was >1.67, a value defined prospectively (3), on two consecutive occasions (27).

Parasitological, Biochemical Observations, and Endothelial Function Assessment. Parasite counts were determined from microscopy of Giemsa-stained thick and thin films. Hemoglobin, routine biochemistry, acid-base parameters (including base deficit), and lactate were measured with a portable biochemical analyzer (i-STAT). Venous blood collected in lithium heparin was centrifuged within 30 min of collection and plasma was stored at −70° C. Plasma concentrations of soluble ICAM-1, E-selectin, Ang-2, and VEGF were measured by ELISA (R&D Systems). Total parasite biomass was quantified by measuring plasma HRP2 by ELISA, as described and validated (3, 26). Plasma TNF concentrations were measured by flow cytometry (BD Cytometric Bead Array), and plasma L-arginine concentrations were measured by HPLC (3). Endothelial function was measured noninvasively by using peripheral arterial tonometry by the change in digital pulse wave amplitude in response to reactive hyperemia, giving a RH-PAT index as described (3). The pulse wave amplitude in RH-PAT is at least 50% dependent

on endothelial NO production (39), and internal validation and repeatability of RH-PAT in this population has been reported (3).

Statistical Methods. Statistical analysis was performed with STATA software (version 9.2; Statacorp). Half the value of the lower limit of detection was used if plasma concentrations of Ang-2 and VEGF were too low to be quantified by ELISA. Results are presented as mean with 95% C.I. for normally distributed continuous variables or median with IQR for those that were not. Intergroup differences were compared by ANOVA for continuous variables normally distributed or that were log-transformed to normality. The Kruskal–Wallis test was used for nonnormally distributed continuous variables. Correlation coefficients were determined by the Pearson's or Spearman's methods for continuous variables, which were normally and not normally distributed, respectively. Multiple stepwise linear regression was conducted to identify confounding variables that could affect the association between markers of disease severity, endothelial function, and Ang-2/VEGF. Linear mixed effects were used to model the longitudinal change in Ang-2 and its association with the RH-PAT index. Logistic regression was used to determine the association between death and Ang-2/VEGF concentrations. Variables hypothesized to contribute to mortality and endothelial pathology were included in a multiple logistic regression model if $P < 0.05$ on univariate analysis and retained if they remained significant. Goodness of fit was assessed by the Hosmer–Lemeshow goodness-of-fit test, and independent variables were tested for interactions. To measure the prognostic utility of continuous variables, the AUROC and its 95% C.I. were calculated. A two-sided value of $P < 0.05$ was considered significant.

ACKNOWLEDGMENTS. We thank Ferryanto Chalfein, Prayoga, Govert Waramori, Betsy Hill, Sri Rahayu, and Tonia Woodberry for technical and logistical assistance; David Sullivan (Johns Hopkins University, Baltimore) for the purified HRP2 protein; Marlini Malisan and Margaretha Ferre for nursing assistance; Mitra Masyarakat Hospital staff for clinical support; Don Granger, Bert Lopansri, Brice Weinberg, Jeanne Rini, Paulus Sugiarto, Chris Engwerda, and Erna Tresnaningsih for advice and support; and Lembaga Pengembangan Masyarakat Amungme Kamoro for support. This study was funded by National Health and Medical Research Council of Australia Grants 283321, 290208, and 496600, Wellcome Trust Grant ICRG GR071614MA, and the Tudor Foundation. R.N.P. is supported by a Wellcome Trust Career Development Award. N.M.A. is supported by a National Health and Medical Research Council Practitioner Fellowship.

1. Beare NA, Taylor TE, Harding SP, Lewallen S, Molyneux ME (2006) Malarial retinopathy: A newly established diagnostic sign in severe malaria. *Am J Trop Med Hyg* 75:790–797.
2. Dondorp AM, *et al.* (2008) Direct in vivo assessment of microcirculatory dysfunction in severe falciparum malaria. *J Infect Dis* 197:79–84.
3. Yeo TW, *et al.* (2007) Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciparum malaria. *J Exp Med* 204:2693–2704.
4. Day NP, *et al.* (2000) The pathophysiologic and prognostic significance of acidosis in severe adult malaria. *Crit Care Med* 28:1833–1840.
5. MacPherson GG, Warrell MJ, White NJ, Looareesuwan S, Warrell DA (1985) Human cerebral malaria: A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol* 119:385–401.
6. Taylor TE, *et al.* (2004) Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nat Med* 10:143–145.
7. Turner GD, *et al.* (1994) An immunohistochemical study of the pathology of fatal malaria: Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. *Am J Pathol* 145:1057–1069.
8. Jakobsen PH, *et al.* (1994) Increased plasma concentrations of sICAM-1, sVCAM-1, and sELAM-1 in patients with *Plasmodium falciparum* or *Plasmodium vivax* malaria and association with disease severity. *Immunology* 83:665–669.
9. Hollestelle MJ, *et al.* (2006) von Willebrand factor propeptide in malaria: Evidence of acute endothelial cell activation. *Br J Haematol* 133:562–569.
10. Combes V, *et al.* (2004) Circulating endothelial microparticles in malawian children with severe falciparum malaria complicated with coma. *J Am Med Assoc* 291:2542–2544.
11. Schofield L, Grau GE (2005) Immunological processes in malaria pathogenesis. *Nat Rev Immunol* 5:722–735.
12. Chakravorty SJ, *et al.* (2007) Altered phenotype and gene transcription in endothelial cells, induced by *Plasmodium falciparum*-infected red blood cells: Pathogenic or protective? *Int J Parasitol* 37:975–987.
13. de Mast Q, *et al.* (2007) Thrombocytopenia and release of activated von Willebrand factor during early *Plasmodium falciparum* malaria. *J Infect Dis* 196:622–628.
14. Fiedler U, Augustin H (2006) Angiopoietins: A link between angiogenesis and inflammation. *Trends Immunol* 27:552–558.
15. Yano K, *et al.* (2006) Vascular endothelial growth factor is an important determinant of sepsis morbidity and mortality. *J Exp Med* 203:1447–1458.
16. Giuliano J, *et al.* (2007) Admission angiopoietin levels in children with septic shock. *Shock* 28:650–654.
17. Fiedler U, *et al.* (2004) The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel–Palade bodies. *Blood* 103:4150–4156.
18. Fiedler U, *et al.* (2006) Angiopoietin-2 sensitizes endothelial cells to TNF-α and has a crucial role in the induction of inflammation. *Nat Med* 12:235–239.
19. Kim I, *et al.* (2001) Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-κB activation in endothelial cells. *J Biol Chem* 276:7614–7620.
20. Oh H, *et al.* (1999) Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem* 274:15732–15739.
21. Casals-Pascual C, *et al.* (2008) High levels of erythropoietin are associated with protection against neurological sequelae in African children with cerebral malaria. *Proc Natl Acad Sci* 105:2634–2639.
22. Matsushita K, *et al.* (2003) Nitric oxide regulates exocytosis by S-nitrosylation of *N*-ethylmaleimide-sensitive factor. *Cell* 115:139–150.
23. Deanfield JE, Halcox JP, Rabelink TJ (2007) Endothelial function and dysfunction: Testing and clinical relevance. *Circulation* 115:1285–1295.
24. Hien TT, *et al.* (1996) Controlled trial of artemether or quinine in Vietnamese adults with severe falciparum malaria. *N Engl J Med* 335:76–83.
25. Davis TM, *et al.* (2001) Pharmacokinetics and pharmacodynamics of intravenous artesunate in severe falciparum malaria. *Antimicrob Agents Chemother* 45:181–186.
26. Dondorp AM, *et al.* (2005) Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med* 2:e204.
27. Yeo TW, *et al.* (2008) Recovery of endothelial function in severe falciparum malaria: Relationship with improvement in plasma L-arginine and blood lactate concentrations. *J Infect Dis* 198:602–608.
28. Rondaij M, Bierings R, Kragt A, van Mourik JA, Voorberg J (2006) Dynamics and plasticity of Weibel–Palade bodies in endothelial cells. *Arterioscler Thromb Vasc Biol* 26:1002–1007.
29. Lowenstein CJ, Morrell CN, Yamakuchi M (2005) Regulation of Weibel–Palade body exocytosis. *Trends Cardiovasc Med* 15:302–308.
30. Into T, *et al.* (2007) Pathogen recognition by Toll-like receptor 2 activates Weibel–Palade body exocytosis in human aortic endothelial cells. *J Biol Chem* 282:8134–8141.
31. Mofarrahi M, *et al.* (2008) Regulation of angiopoietin expression by bacterial lipopolysaccharide. *Am J Physiol* 294:L955–L963.
32. Orfanos S, *et al.* (2007) Angiopoietin-2 is increased in severe sepsis: Correlation with inflammatory mediators. *Crit Care Med* 35:199–206.
33. Gowda DC (2007) TLR-mediated cell signaling by malaria GPIs. *Trends Parasitol* 23:596–604.
34. Schofield L, *et al.* (1996) Glycosylphosphatidylinositol toxin of *Plasmodium* up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. *J Immunol* 156:1886–1896.
35. Tripathi AK, Sullivan DJ, Stins MF (2006) *Plasmodium falciparum*-infected erythrocytes increase intercellular adhesion molecule 1 expression on brain endothelium through NF-κB. *Infect Immun* 74:3262–3270.
36. MacDonald SM, *et al.* (2001) Immune mimicry in malaria: Plasmodium falciparum secretes a functional histamine-releasing factor homolog in vitro and in vivo. *Proc Natl Acad Sci USA* 98:10829–10832.
37. Day NP, *et al.* (1999) The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J Infect Dis* 180:1288–1297.
38. Anstey NM, *et al.* (1996) Nitric oxide in Tanzanian children with malaria: Inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J Exp Med* 184:557–567.
39. Nohria A, *et al.* (2006) Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *J Appl Physiol* 101:545–548.
40. Yipp BG, *et al.* (2007) Differential roles of CD36, ICAM-1, and P-selectin in *Plasmodium falciparum* cytoadherence in vivo. *Microcirculation* 14:593–602.
41. Combes V, *et al.* (2004) Pathogenic role of P-selectin in experimental cerebral malaria: Importance of the endothelial compartment. *Am J Pathol* 164:781–786.
42. Facer CA, Theodoridou A (1994) Elevated plasma levels of P-selectin (GMP-140/CD62P) in patients with *Plasmodium falciparum* malaria. *Microbiol Immunol* 38:727–731.
43. Jain V, *et al.* (2008) Plasma IP-10, apoptotic, and angiogenic factors associated with fatal cerebral malaria in India. *Malar J* 7:83.
44. Sachanonta N, *et al.* (2008) Host vascular endothelial growth factor is trophic for *Plasmodium falciparum*-infected red blood cells. *Asian Pac J Allergy Immunol* 26:37–45.
45. Ziche M, *et al.* (1997) Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 99:2625–2634.
46. World Health Organization (2000) Severe falciparum malaria. *Trans R Soc Trop Med Hyg* 94(Suppl 1):S1–S90.
47. Grau GE, *et al.* (1989) Tumor necrosis factor and disease severity in children with falciparum malaria. *N Engl J Med* 320:1586–1591.
48. Matsushita K, Morrell CN, Lowenstein CJ (2005) A novel class of fusion polypeptides inhibits exocytosis. *Mol Pharmacol* 67:1137–1144.
49. Oliner J (2004) Suppression of angiogenesis and tumor growth by selective inhibition of Angiopoietin-2. *Cancer Cell* 6:507–516.
50. Ratcliff A, *et al.* (2007) Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in Papua, Indonesia: An open-label randomized comparison. *Lancet* 369:757–765.
51. Karyana M, *et al.* (2008) Malaria morbidity in Papua Indonesia, an area with multidrug resistant *Plasmodium vivax* and *Plasmodium falciparum*. *Malaria J* 7:148.